Structural elucidation of novel phosphocholine-containing glycosylinositol-phosphoceramides in filamentous fungi and their induction of cell death of cultured rice cells

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Novel ZGLs (zwitterionic glycosphingolipids) have been found in and extracted from the mycelia of filamentous fungi (*Acremonium* sp.) isolated from soil. Five ZGLs (ZGL1–ZGL5) were structurally elucidated by sugar compositional analysis, methylation analysis, periodate oxidation, matrix-assisted laser-desorption ionization–time-of-flight MS, ^{1}H-NMR spectroscopy and fast-atom bombardment MS. Their chemical structures were as follows: GlcN(α1-2)Ins1-*P*-1Cer (ZGL1), Man(α1-6)GlcN(α1-2)Ins1-*P*-1Cer (ZGL2), Man(α1-6)Man(α1-6)GlcN(α1-2)Ins1-*P*-1Cer (ZGL3), PC $\rightarrow$ 6Man(α1-6)GlcN(α1-2)Ins1-*P*-1Cer (ZGL4), and PC $\rightarrow$ 6Man(α1-6)Man(α1-6)GlcN(α1-2)Ins1-*P*-1Cer (ZGL5) (where Cer is ceramide and PC is phosphocholine). In addition, one acidic glycosphingolipid, which was the precursor of ZGLs, was also characterized as inositol-phosphoceramide. The core structure of the ZGLs, GlcN(α1-2)Ins1-*P*, is rather different from those found in other fungi, such as Man(α1-2)Ins1-*P* and Man(α1-6)Ins1-*P*. Interestingly, the terminal mannose residue of ZGL4 and ZGL5 was modified further with a PC group. The presence of PC-containing glycosylinositol-phosphoceramides has not been reported previously in any organism. The ceramide constituents of both ZGLs and acidic glycosphingolipid were essentially the same, and consisted of a 4-hydroxyoctadecasphinganine (phytosphingosine) as the sole sphingoid base and 2-hydroxytetracosanoic acid (>90 %) as the major fatty acid. ZGLs were found to cause cell death in suspensions of cultured rice cells. The cell death-inducing activity of ZGLs is probably due to the characteristic glycan moiety of Man(α1-6)GlcN, and PC-containing ZGLs had high activity. This study is the first to demonstrate that fungal glycosylinositol-phosphoceramides induce cell death in cultured rice cells.

Key words: cell death, elicitor, phosphocholine, plant–pathogen interaction, rice cell, zwitterionic glycosphingolipid.

INTRODUCTION

Sphingolipids are ubiquitous and important components of the eukaryotic plasma membrane. They play important roles in membrane permeability and fluidity, and act as second messengers in response to various stress factors that are involved in cell cycle control, calcium homoeostasis, apoptosis, etc. [1–4]. The structure of sphingolipids is different in fungal and mammalian cells. Fungal sphingolipids consist of phytosphingosine in the ceramide moiety instead of sphingosine, which is mainly present in mammals. The biosynthetic pathway for sphingolipids also varies in fungal and mammalian cells [5]. In all fungi, inositol phosphate is added to phytoceramide to form IPC (inositol-phosphoceramide), while in mammals sugar is added to ceramide to form glycosylceramide. Among various fungi, only yeast (*Saccharomyces cerevisiae*) has been well characterized with regard to the pathway for sphingolipids, and many genes encoding enzymes involved in this pathway have been cloned [6]. The major sphingolipids in *S. cerevisiae* are the IPC family members, including IPC, MIPC (mannose-IPC) and M(IP)$_2$C [mannose-(di-inositol-phospho)ceramide]. Their phytoceramides generally consist of 4-hydroxysphinganine as the sphingoid base bound to a long-chain fatty acid with its 2-amino group as the amide linkage. These are subjected to *myo*-inositol phosphate addition, and then mannosylated to form MIPC. M(IP)$_2$C is finally formed through the addition of *myo*-inositol phosphate to MIPC, which is the major sphingolipid in *S. cerevisiae* [7]. Sphingolipid synthesis is necessary for the growth and viability of *S. cerevisiae*, and this is demonstrated by the fact that the addition of aureobasidin A, a potent inhibitor of IPC synthase (which catalyses the transfer of *myo*-inositol 1-phosphate from phosphatidylinositol to phytoceramide), leads to the accumulation of phytoceramide and induces cell death [8]. Although there have been many reports about the important roles that the sphingolipids play in *S. cerevisiae* [9–11], not only the structures and biosynthetic pathway but also the roles and functions of the sphingolipids of filamentous fungi remain unknown.

Recently, a few papers have demonstrated the *in vitro* physiological activities of fungal cerebroside (ceramide monosaccharide) [12]. The fungal cerebroside or structurally similar analogues showed fruiting-inducing activity in *Schizophyllum commune* [13], and cerebroside extracted from the rice pathogen *Magnaporthe grisea* was found to exhibit high elicitor activity that elicited various defence responses in plants [14,15]. These responses cause the accumulation of phytoalexins and the hypersensitive cell death of the plant. In addition, phospholipomannan,

Abbreviations used: AGL, acidic glycosphingolipid; FAB/MS, fast-atom bombardment MS; GIPC, glycosylinositol-phosphoceramide; GPI, glycosylphosphatidylinositol; GSL, glycosphingolipid; IPC, inositol-phosphoceramide; MALDI-TOF/MS, matrix-assisted laser-desorption ionization–time-of-flight MS; MIPC, mannose-inositol-phosphoceramide; M(IP)$_2$C, mannose-(di-inositol-phospho)ceramide; NAc-ZGL N-acetylated zwitterionic glycosphingolipid; NGL, neutral glycosphingolipid; PC, phosphocholine; ZGL, zwitterionic glycosphingolipid.

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one of the GSLs (glycosphingolipids) of the pathogenic yeast *Candida albicans*, has been reported to act as an adhesive to induce cytokine production [16,17]. Although there are many filamentous fungi that cause mucocutaneous, asthmatic and allergic reactions [18], such as *C. albicans*, almost nothing is known about their GSLs. Considering these facts, more detailed studies on filamentous fungi are required, because information is still limited in comparison with that on yeast and mammalian GSLs [5,19].

In the present paper, we elucidate the structures of novel GIPCs (glycosylinositol-phosphoceramides) found in *Acremonium* and *Trichoderma*, and also indicate their ability to induce the cell death of cultured rice cells. Interestingly, an additional PC (phosphocholine) group was bound to the terminal mannose of the oligosaccharide moiety in some glycolipids, and such GSLs had higher cell death-inducing activity. *Mycoplasma fermentans*, which is involved in several pathogenic conditions in humans, is known to have PC-containing glycoglycerolipids [20]. Moreover, PC-containing macromolecules have been detected in the extracts of numerous parasitic nematode species [21,22]. These findings suggest that PC-containing glycoconjugates are important mediators of tissue pathology in an infectious process [23,24].

To our knowledge, this is the first report of detection of the presence of PC-containing GIPCs in fungal cells. We also show that the oligosaccharide core structures of these GIPCs are different from those of yeast and non-pathogenic fungi, but are similar to that of GPI (glycosylphosphatidylinositol)-anchored proteins in animals [25].

EXPERIMENTAL

Strains and growth conditions

Fungal strains *Aspergillus oryzae* (IFO4075), *Acremonium bacillisporum* (IFO9387), *Acremonium terricola* (IFO30538), *Mucor fragilis* (IFO6449), *Mucor racemosus* (IFO4581), *Rhizopus oryzae* (IFO5441) and *Trichoderma viride* (IFO30498) were obtained from the Institute for Fermentation Osaka (IFO, Osaka, Japan). *Acremonium* sp. no. 413 was isolated from soil and identified in our laboratory [26]. These strains were maintained on YPD medium (0.5 % yeast extract/0.5 % peptone/0.5 % NaCl/1 % glucose, pH 6.5) or potato-glucose medium. For cultivation, a loopful of mycelia was taken from a slant, inoculated into 5 ml of liquid medium in a test tube, and then cultivated at 28 °C for 72 h with shaking. This seed culture was then transferred to a 2 litre shaking flask containing 700 ml of the medium, and cultivation was carried out at 28 °C on a reciprocal shaker at 120 rev./min.

Materials

QAE-Sephadex A-25 and DEAE Sephadex A-25 were purchased from Amersham Pharmacia Biotech. Iatrobeads 6RS-8060 were obtained from Iatron Lab. Inc. Silica gel 60-precoated plates were from Merck. Magnesium silicate (Florisil) was from Nacalai Tesque. Jackbean α-mannosidase and *Achatina fulica* β-mannosidase were from Seikagaku Co. *myo*-Inositol 1-monophosphate and alkaline phosphatase (*Escherichia coli*) were obtained from Sigma Chemical Co. All other reagents used were of guaranteed grade available commercially.

Extraction and fractionation of GSLs from *Acremonium* sp.

The cultivated mycelia were collected, suspended in acetone, and then filtered to dryness. The mycelia obtained (190 g) were extracted twice with 2.1 litres of chloroform/methanol (2:1, v/v), and then extracted further with 2 litres of chloroform/methanol/water (30:60:8, by vol.) to recover hydrophilic lipids. The extracts were combined and concentrated by evaporation at 40 °C. The concentrated extract was subjected to mild alkaline hydrolysis with 0.5 M KOH in methanol/water (95:5, v/v) at 37 °C for 6 h to eliminate glycerolipids. The hydrolysate was acidified to pH 1.0 with conc. HCl and then dialysed against tap water for 2 days, followed by concentration and precipitation with acetone. The resulting precipitate (4.0 g) of the alkali-stable product was dissolved in chloroform/methanol/water (30:60:8, by vol.) and then applied to a QAE-Sephadex A-25 column (bed volume 300 ml; OH^- form). Elution was performed with 5 vol. of the above solution to obtain the NGL (neutral GSL) fraction, with the polar compounds being recovered with 5 vol. of 0.45 M ammonium acetate in methanol. The polar compounds were concentrated to approx. 100 ml, and then dialysed against water to remove acetate. These compounds were then applied to a column of DEAE-Sephadex A-25 (bed volume 300 ml; acetate form) and eluted with 5 vol. of chloroform/methanol/water (30:60:8, by vol.) to obtain the ZGL (zwitterionic GSL) fraction. Thereafter, the AGL (acidic GSL) fraction was eluted with 5 vol. of 0.45 M ammonium acetate in methanol, and then dialysed against water to remove acetate. The lipid extracts of other fungi were fractionated using the same procedure.

Purification of *Acremonium* sp. GSLs

The ZGL fraction obtained by the above method was applied to a column of porous silica gel 6RS-8060 (2.0 cm × 60 cm) equilibrated with a propan-1-ol/water/ammonia solution (75:15:5, by vol.). The column was eluted with the same solvent and then with a solvent system of propan-1-ol/water/ammonia solution (75:30:5, by vol.). Fractions of 5 ml were collected, and 5 μl aliquots from alternate tubes were analysed by TLC. The NGL fraction was applied to a column of porous silica gel 6RS-8060 (2.0 cm × 60 cm) with a linear gradient elution system of chloroform/methanol/water [400 ml of 90:10:0.5 (by vol.) to 420 ml of 80:20:1 (by vol.)]. The AGL fraction was applied to the same silica gel column (1.2 cm × 60 cm) equilibrated with propan-1-ol/water/ammonia solution (75:5:5, by vol.), and eluted with the same solvent mixture.

Analytical procedures

For determination of the composition of fatty acids and sugars in glycolipids, 100–200 μg of glycolipid was methanolysed in thick glass test tubes (16 mm × 125 mm, with Teflon-lined screw caps; Pyrex, Iwaki Glass Co.) with 200 μl of freshly prepared 1 M anhydrous methanolic HCl, using a microwave oven (Sharp RE-Z3W6, 100 V, 60 Hz; Sharp Electric Co.). The samples were exposed to the maximum power (500 W) of the microwave oven for 1 min [27]. After methanolysis, the samples were cooled to room temperature. The fatty acid methyl esters produced were extracted three times with 400 μl of n-hexane, and then analysed using a Shimadzu GC-18A gas chromatograph with a Shimadzu HiCap-CBP 5 capillary column (0.22 mm × 25 m). The temperature programme was 2 °C/min from 170 °C to 230 °C. The methanolic phase remaining was evaporated to dryness for deacidification under a nitrogen stream. The residue containing methylglycosides was trimethylsilylated with pyridine/hexamethyldisilazane/trimethylchlorosilane (9:3:1, by vol.). The reaction mixture was analysed by GLC on the capillary column described above using a temperature programme of 2 °C/min from 140 °C to 230 °C. Sphingoids prepared from glycolipids by methanolysis with 1 M aqueous methanolic HCl at 70 °C for

18 h were converted into their *O*-trimethylsilyl (nitrogen-free) derivatives and then analysed by GLC on the same capillary column using a temperature programme of 2 °C/min from 210 °C to 230 °C. For determination of sugar linkages, 300 μg of each purified glycolipid was partially methylated with NaOH and methyl iodide in DMSO according to a modification of the method of Ciucanu and Kerek [28]. The permethylated glycolipid was acetolysed and hydrolysed with 300 μl of HCl/water/acetic acid (0.5:1.5:8, by vol.) by exposure to the maximum power of the microwave oven for 1 min, and then reduced with $NaBH_4$ and acetylated with acetic anhydride/pyridine (1:1, v/v) at 100 °C for 15 min. The partially methylated alditol acetates thus obtained were analysed by GLC and GLC/MS equipped with the same HiCap-CBP 5 capillary column as described above. Electron impact and chemical ionization mass spectra were obtained using a Shimadzu GCMS-QP 5050 GLC/MS instrument under the following conditions: oven temperature, 80 °C (2 min) → 180 °C (20 °C/min) → 240 °C (4 °C/min); interface temperature, 250 °C; injection port temperature, 240 °C; helium gas pressure, 100 kPa; ionizing voltage, 70 eV (electron impact) and 100 eV (chemical ionization); ionizing current, 60 μA (electron impact) and 200 μA (chemical ionization); reaction gas (chemical ionization), iso-butane.

Pretreatment of ZGLs for analyses

To analyse the sugar compositions of purified ZGLs, the compounds were N-acetylated prior to acid hydrolysis because of the strong linkage between hexosamine and other sugars [29]. The ZGLs were acetylated with acetic anhydride/pyridine (3:2, v/v) at 37 °C for 12 h, and then O-deacetylated with 0.5 M KOH in methanol at 37 °C for 6 h. After dialysis of the reaction mixture against tap water and lyophilization, the NAc-ZGLs (N-acetylated ZGLs) were hydrolysed with 2 M HCl at 100 °C for 20 h, and then the alditol acetate derivatives of sugar components in the hydrolysates were prepared and subjected to GLC.

Degradation with HF

For selective cleavage of phosphate bonds, each of the purified AGLs and ZGLs was treated with HF [30]. To 0.2 ml of DMSO containing approx. 500 μg of sample in a polyethylene tube was added 3.5 ml of 47 % (w/v) HF, and then the mixture was allowed to stand for 20 h at 15 °C. After that, the reaction mixture was dialysed against tap water to remove HF, and then lyophilized. The dephosphorylated lipid was purified by Iatrobead column (0.5 cm × 8 cm) chromatography using chloroform/methanol (95:5, v/v) as the eluting solvent system.

Inositol phosphate analysis

To confirm the inositol phosphate content of GSLs, a purified AGL was hydrolysed with 2 M HCl at 100 °C for 100 min and then dried *in vacuo*. The residue was subjected to Folch partitioning, and the upper phase containing the water-soluble component was dried and then analysed by GLC and GLC/MS after trimethylsilylation with bis(trimethylsilyl)acetamide/trimethylchlorosilane/pyridine (10:2:5, by vol.) at 60 °C for 1 h.

TLC

TLC was performed using silica gel 60-precoated plates, with a neutral solvent system of chloroform/methanol/water (60:40:10, by vol.) and a basic solvent system of propan-1-ol/water/ammonia solution (75:25:5 and 75:30:5, by vol.). Detection was performed by spraying with orcinol/H_2SO_4 reagent for sugars, 5 % H_2SO_4/ethanol reagent for organic substances, Dittmer–Lester reagent and Hanes–Isherwood reagent for phosphorus, ninhydrin reagent for free amino groups, and Dragendorff's reagent for choline.

Determination of linkages between sugars and inositol

A modification of the procedure of Hsieh et al. [31] and Sugita et al. [32] was used to determine the linkages of sugars with inositol. A 4 ml portion of 80 mM $NaIO_4$ in 0.2 M sodium acetate buffer (pH 4.0) was added to 1 ml of an ethanol solution containing approx. 5 mg of purified ZGL. The oxidative reaction was allowed to proceed in the dark at 4 °C for 120 h. The reaction was stopped by adding 500 μl of ethylene glycol to destroy the excess $NaIO_4$, and then made alkaline with 1 ml of 3 M ammonia solution. To reduce the oxidized product, 1 ml of 5 % $NaBH_4$ in 1 M ammonia solution was added, and the mixture was allowed to react at room temperature for 12 h. After acetic acid had been added to destroy excess $NaBH_4$, the reaction mixture was dialysed against tap water and lyophilized. The periodate oxidation products were subjected to methanolysis. After extraction of fatty acid methyl esters with 1-hexane, the phosphoalcohol in the hydrolysate was separated from other oxidation products by anion-exchange column chromatography (Dowex 1 × 8, Cl^- form, 100–200 mesh; column 0.5 cm × 4 cm) using 100 ml of water and 100 ml of 0.1 M HCl as the eluting solvents. The fractions eluted with HCl were evaporated to dryness, and the phosphate group was removed by incubating the product with 2 units of alkaline phosphatase in 0.2 M ammonium acetate buffer (pH 8.5) at 37 °C for 24 h. After incubation, the reaction mixture was dried under a stream of N_2, acetylated, and then analysed by GLC and GLC/MS. The resulting peaks on the gas chromatograms were identified by MS by comparison with the retention times of the following reference alditol acetates: glycerol, erythritol, threitol, ribitol, arabitol, xylitol, alitol, inositol, altriol, glucitol and iditol.

Cleavage of sugar linkages by exoglycosidases

α-Mannosidase from jackbean and β-mannosidase from *Ach. fulica* were used for exoglycosidase cleavage of sugar linkages. Samples (10–30 μg) were suspended in 0.1 ml of 50 mM citrate buffer (pH 4.5) containing 0.1 mg of sodium taurodeoxycholate for α-mannosidase treatment, or in 100 mM citrate buffer (pH 4.5) for β-mannosidase treatment. The reaction was carried out with 0.1 unit of α-mannosidase or 0.01 unit of β-mannosidase at 37 °C for 12 h. Each reaction was stopped by adding 0.5 ml of chloroform/methanol (2:1, v/v). The hydrolysates, after extraction into the lower phase, were dried under a nitrogen stream.

^{1}H-NMR spectroscopy

NMR spectra of purified ZGLs were obtained using a JEOL A-500 500 MHz ^{1}H-NMR spectrometer at an operating temperature of 60 °C. The purified glycolipid was dissolved in 0.60 ml of [$^2H_6$]DMSO containing 2 % ^2H_2O, and chemical shift was referenced to the solvent signals (δ_H 2.49 p.p.m.) in [$^2H_6$]DMSO as the internal standard.

FAB/MS (fast-atom bombardment MS)

FAB/MS analyses of purified ZGLs were performed using a JEOL HX-110 mass spectrometer equipped with a JMA-DA 5000 computer system, operating in the negative-ion mode. Triethanolamine was used as the matrix. The accelerating voltage

was 8.0 kV, and the primary beam for the bombardment was 6.0 keV xenon.

MALDI-TOF/MS (matrix-assisted laser-desorption ionization–time-of-flight MS)

MALDI-TOF/MS analyses of the purified GSLs were performed using a Shimadzu/KRATOS KOMPACT MALDI I mass spectrometer equipped with a Workstation SPARC station, operating in the negative-ion linear mode. Ions were formed by a pulsed UV laser beam (N_2 laser, 337 nm; 3 ns-wide pulses/s). The matrix used was 7-amino-4-methylcoumarin (Sigma Chemical Co.) [33]. External mass calibration was provided by the $[M-H]^-$ ions of angiotensin II (1046.2 mass units; Sigma Chemical Co.) and neurotensin-(1–11) (1446.6 mass units; Sigma Chemical Co.).

Cell death assay of cultured rice cells

Suspension cultures of rice cells (Oc line) were grown in 100 ml Erlenmeyer flasks containing 20 ml of R2S medium [34] at 30 °C under light on a rotary shaker at 107 rev./min. The cells were diluted in fresh medium every 7 days. For the assay, 10 ml of a suspension culture grown for 4 days was transferred to a 50 ml flask and a glycolipid dissolved in DMSO was added. Then the cultured cells were incubated further with shaking at 90 rev./min, and 1 ml portions of the culture were collected and put in a 24-well tissue culture plate at appropriate time points. The supernatants were removed and the cells were stained with 0.5 ml of 0.05 % Evans Blue containing 50 mM Hepes/KOH buffer (pH 7.2) for 15 min. The cells were then washed three times with 2 ml of distilled water to remove excess dye. The dye incorporated into dead cells was extracted with 50 % (v/v) methanol containing 1 % SDS for 12 h at room temperature [34]. The concentration of the extracted dye was determined by measuring the absorbance at 595 nm.

RESULTS

Analyses of GSLs from various filamentous fungi

GSLs of the mycelia of various filamentous fungi were analysed. At first, the following typical strains were examined: *Asp. oryzae* (IFO4075), *Acr. bacillisporum* (IFO9387), *Acr. terricola* (IFO30538), *M. fragilis* (IFO6449), *M. racemosus* (IFO4581) and *R. oryzae* (IFO5441). Lipids extracted from fungal mycelia were submitted to mild alkaline hydrolysis, and the resulting alkali-stable substances, including GSLs, were fractionated into three fractions (i.e. neutral, zwitterionic and acidic components), based on their polarities on ion-exchange column chromatography. Each fraction was analysed by TLC with a chloroform/methanol/water system. The GSLs of the filamentous fungi were recovered in the AGL and/or NGL fractions, but not in the ZGL fraction. The AGLs obtained seemed to be inositol-containing sphingolipids such as MIPC, which are generally found in fungal cells and *S. cerevisiae* [5,19], and the NGL seemed to be glucosylceramide [35]. On the other hand, *Acremonium* sp. no. 413, which was isolated in our laboratory, contained NGLs and ZGLs as major components. The analysis of various fungal GSLs is summarized in Table 1. The presence of ZGLs has not been reported previously in any filamentous fungi. Therefore the GSLs of *Acremonium* sp. 413 were analysed in the present study. As described below, *T. viride*, a plant pathogenic fungus, contained ZGLs which were confirmed to be exactly the same as the major components of ZGLs from *Acremonium* sp. by MALDI-TOF/MS and GLC/MS analyses.

Table 1 GSLs present in various filamentous fungi

Fungal GSLs were fractionated into NGLs, AGLs and ZGLs by ion-exchange column chromatography, based on their polarities. Each glycolipid was analysed by TLC with the reagents orcinol/H_2SO_4, Dittmer–Lester reagent, Hanes–Isherwood reagent and ninhydrin. The yields are indicated as: +, presence; −, absence; ±, scarcely detected.

Fungal strain	NGL	AGL	ZGL
Aspergillus oryzae	+	+	−
Acremonium bacillisporum	+	+	−
Acremonium terricola	+	+	−
Mucor fragillis	+	±	−
Mucor racemosus	+	±	−
Rhizopus oryzae	+	±	−
Acremonium sp. no. 413	+	+	+

Figure 1 TLC of fractionated GSLs from *Acremonium* sp.

TLC was performed with chloroform/methanol/water (60:40:10, by vol.; neutral solvent), with visualization with orcinol/H_2SO_4 reagent. Lane T, whole GSLs from *Acremonium* sp.; lanes 1–3, NGL, ZGL and AGL fractions respectively from *Acremonium* sp.

Isolation and characterization of GSLs from *Acremonium* sp.

Acremonium sp. 413 was cultivated, the lipids extracted from the dried mycelia (190 g) were treated with alkaline hydrolysis, and the resulting alkali-stable substances (4.0 g) were fractionated into NGL (0.7 g), ZGL (1.1 g) and AGL (20 mg) fractions by ion-exchange column chromatography. As shown in Figure 1, the major GSLs obtained were NGL and ZGL, while AGL was barely detected by orcinol/H_2SO_4 reagent. The NGL was purified and analysed by various methods, including GLC and GLC/MS analyses, and its structure was determined to be glucosylceramide containing the fatty acids 2-hydroxyhexadecanoic acid ($C_{h16:0}$; (58 %) and 2-hydroxytetracosanoic acid ($C_{h18:0}$; 42 %), as well as 9-methyl-4,8-sphingadienine (results not shown) [14,15,35].

Next, the ZGL fraction, which contained several compounds, was analysed further by TLC and successfully separated into five components, namely ZGL1, ZGL2, ZGL3, ZGL4 and ZGL5, using a propan-1-ol/water/ammonia system (Figure 2). These purified ZGLs showed positive reactions with orcinol/H_2SO_4 reagent (Figure 2A), Dittmer–Lester reagent, Hanes–Isherwood reagent (Figure 2B) and ninhydrin reagent, although ZGL1 showed a negative reaction with orcinol/H_2SO_4 reagent. Interestingly, ZGL4 and ZGL5 reacted slightly with Dragendorff's reagent. IR spectra suggested that all of the ZGLs contained a sugar residue, a ceramide moiety and phosphorus, and both ZGL4 and ZGL5 showed sharp absorbance at around 960 cm^{-1}, which is attributable in part to a choline residue (results not shown)

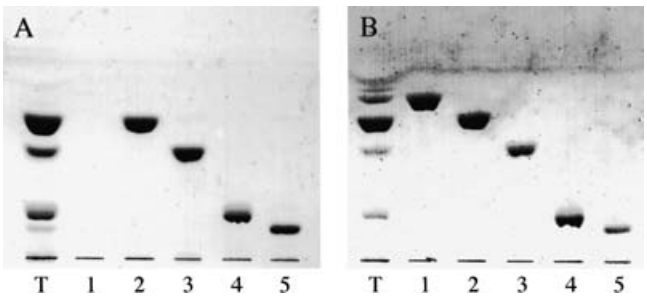


Figure 2 TLC of purified ZGLs from *Acremonium* sp.

Purified ZGLs from *Acremonium* sp. were subjected to TLC with propan-1-ol/water/15 M NH_4OH (75:30:5, by vol.; basic solvent), with visualization with orcinol/H_2SO_4 reagent (**A**) and Hanes–Isherwood reagent (**B**). Lane T, ZGL fraction from *Acremonium* sp.; lanes 1–5, purified ZGL1, ZGL2, ZGL3, ZGL4 and ZGL5 respectively.

[36]. There have been no previous reports that choline-containing glycolipids exist in fungi, including yeast and mushroom. The yields of the purified ZGLs were: ZGL1, 45 mg; ZGL2, 138 mg; ZGL3, 16 mg; ZGL4, 39 mg; ZGL5, 5 mg. As all of the ZGLs were confirmed to contain phosphate (Figure 2B); the molar proportion of phosphate in each glycolipid was determined by the method of King [37], giving values of 0.88 for ZGL1, 0.97 for ZGL2, 0.91 for ZGL3, 1.75 for ZGL4 and 1.83 for ZGL5 based on their molecular masses, which were calculated by the MALDI-TOF/MS analyses described below. This indicated the presence of one molecule of phosphate in ZGL1, ZGL2 and ZGL3, and two molecules of phosphate in ZGL4 and ZGL5.

In addition, we also analysed by TLC the AGL that showed a negative reaction with orcinol/H_2SO_4 reagent (Figure 1). One positive spot was faintly detected with molybdate reagent and 5% H_2SO_4/ethanol. Therefore we purified AGL (yield 2 mg) by Iatrobeads column chromatography. The molar proportion of its phosphate residue was determined to be 0.92, and the dephosphorylation product of the AGL on HF treatment migrated to a position corresponding to an authentic ceramide on TLC. Moreover, this dephosphorylation product was also analysed by MALDI-TOF/MS, and its spectra revealed pseudomolecular ions (571.3 *m*/*z* and 683.1 *m*/*z*) of the ceramide (results not shown). These observations suggested that the phosphate residue is linked to the ceramide in AGL. The phosphorus compound was determined to be *myo*-inositol 1-monophosphate by GLC/MS (results not shown) [38]. From these results, AGL was confirmed to be IPC. Although IPC is an important precursor of GIPCs such as MIPC, this is, to the best of our knowledge, the first report of the presence of free IPC in filamentous fungi.

Aliphatic composition of ZGLs and AGL

The aliphatic components of ZGLs and AGL were determined by GLC and GLC/MS. Identifications were accomplished by comparison with the mass spectra of authentic standards. The main fatty acid of ceramide in the ZGLs and AGL was 2-hydroxytetracosanoic acid, while the minor fatty acid component 2-hydroxyhexadecanoic acid was also present in AGL, ZGL1, ZGL2 and ZGL3 (Table 2). The sphingoid was composed entirely of 4-hydroxyoctadecasphinganine (phytosphingosine) in both ZGLs and AGL. Such phytoceramides consisting of a 2-hydroxy fatty acid and phytosphingosine are usually found in fungal cells [5,19]. As the ceramide compositions of both the ZGLs and AGL were the same, AGL is assumed to be the precursor of the ZGLs.

Table 2 Aliphatic compositions of the purified GSLs from *Acremonium* sp.

h16:0, 2-hydroxyhexadecanoic acid; h24:0, 2-hydroxytetracosanoic acid; t18:0, 4-hydroxysphinganine. tr, trace.

	Composition (%)					
Constituent	AGL	ZGL1	ZGL2	ZGL3	ZGL4	ZGL5
Fatty acid						
h16:0	7.7	3.4	3.5	5.9	tr.	tr.
h24:0	92.3	96.6	96.5	94.1	> 99	> 99
Sphingoid						
t18:0	100	100	100	100	100	100

Sugar component analysis of ZGLs

MALDI-TOF/MS analyses were carried out on ZGLs, and the highest pseudomolecular ions were found at *m*/*z* 1087.5 for ZGL1, *m*/*z* 1249.0 for ZGL2, *m*/*z* 1411.3 for ZGL3, *m*/*z* 1414.7 for ZGL4 and *m*/*z* 1577.2 for ZGL5 (these results are described in detail below). The differences in *m*/*z* values between ZGL1 and ZGL2, and between ZGL2 and ZGL3, correspond to a molecular mass of 162 Da, which is attributable to a hexose residue. Similarly, the difference in molecular mass of 162 Da between ZGL4 and ZGL5 was also attributed to an additional hexose residue. On the other hand, the difference in molecular mass of 166 Da between ZGL2 and ZGL4, and between ZGL3 and ZGL5, is thought to be caused by the addition of PC [-PO(OH)-O-CH_2-CH_2-N(CH_3)$_3$] to ZGL2 and ZGL3. These findings indicated that ZGL2 and ZGL3 are glycosylated ZGL1 with different numbers of hexoses, and that both ZGL4 and ZGL5 are phosphorylated and glycosylated ZGL1 with PC.

In order to determine the sugar components of ZGLs, compositional analysis of ZGL1 was first carried out by the methanolysis method. However, no sugar peak was detected in the gas chromatograms. We concluded that the oligosaccharide of ZGL1 was not degraded by acid hydrolysis and therefore is likely contain an amino sugar such as hexosamine, since the strong binding of hexosamine to another sugar is resistant to acid hydrolysis, and ZGL1 also showed a positive reaction with ninhydrin reagent (but not orcinol/H_2SO_4 reagent) on TLC. Accordingly, these ZGLs were N-acetylated and the resulting NAc-ZGLs were hydrolysed completely with 2 M HCl at 100 °C for 20 h [29,32]. Consequently, the alditol acetate derivatives of the ZGL1 hydrolysate were analysed by GLC, and sugar components could be determined as *N*-acetylglucosamine and inositol by comparison with the mass spectra and retention times of the authentic carbohydrates. Additionally, in order to determine the sugar residue bound with phosphorus of ZGL1, which reacted with molybdate reagent, the trimethylsilyl derivatives of the partial acid hydrolysate of ZGL1 were analysed by GLC/MS, and the mass spectrum was assigned to a heptatrimethylsilyl derivative prepared from authentic *myo*-inositol 1-monophosphate (results not shown) [38]. From these observations, it was confirmed that ZGL1 is composed of glucosamine and IPC. Other NAc-ZGLs prepared from ZGL2, ZGL3, ZGL4 and ZGL5 were also analysed as above, revealing that these GSLs contain not only glucosamine and *myo*-inositol 1-monophosphate as sugar components, but also mannose. As shown in Table 3, all ZGLs contained both inositol and glucosamine as common components; they also all contained mannose except for ZGL1. The molar proportions of the sugar components of ZGL2 and ZGL4 were the same as those of ZGL3 and ZGL5 respectively. Therefore the differences in the R_f values of ZGL2 and ZGL4, and of ZGL3 and ZGL5, on TLC seem to be due to the presence or absence of a choline phosphate.

Table 3 Sugar compositions of the purified glycolipids from *Acremonium* sp.

The alditol acetate derivatives of each glycolipid were analysed by GLC and GLC/MS, and the results are expressed relative to Ins (= 1.00). The presence of Ins in AGL is indicated by +.

	Relative amount					
Constituent	AGL	ZGL1	ZGL2	ZGL3	ZGL4	ZGL5
Ins	+	1.00	1.00	1.00	1.00	1.00
GlcN	–	1.11	1.12	1.09	1.05	1.11
Man	–	–	1.03	1.94	0.95	1.89

TLC of the partial hydrolysates of intact ZGL4 and ZGL5 (2 M HCl at 100 °C for 1 h) revealed the presence of mannose monophosphate, mannose (hydrolysate of mannose monophosphate), *myo*-inositol monophosphate and choline. The phosphorylated compounds were trimethylsilylated and then subjected to GLC/MS, and the mass spectra obtained were identified as those of heptatrimethylsilyl derivatives of *myo*-inositol 1-monophosphate and hexatrimethylsilyl derivatives of mannose 6-monophosphate by comparison with authentic standards (results not shown). This observation indicated that ZGL4 and ZGL5 contain both *myo*-inositol 1-monophosphate and mannose 6-monophosphate as phosphorylated compounds in their glycolipid molecules.

Anomeric configuration analyses of sugar components in ZGLs

For determination of the anomeric configurations of the sugar residues, ZGLs were subjected to ^{1}H-NMR spectroscopy (Figure 3). In the anomeric region of the spectrum for each ZGL, the following anomeric proton resonances were observed: at 5.13 p.p.m. (J 3.7 Hz), demonstrating α-glucosamine, for ZGL1 (Figure 3A); at 5.18 p.p.m. (J 3.7 Hz), demonstrating α-glucosamine, and at 4.65 p.p.m. (J 1.2 Hz), demonstrating α-mannose, for ZGL2 (Figure 3B); at 4.63 p.p.m. (J 1.2Hz) and

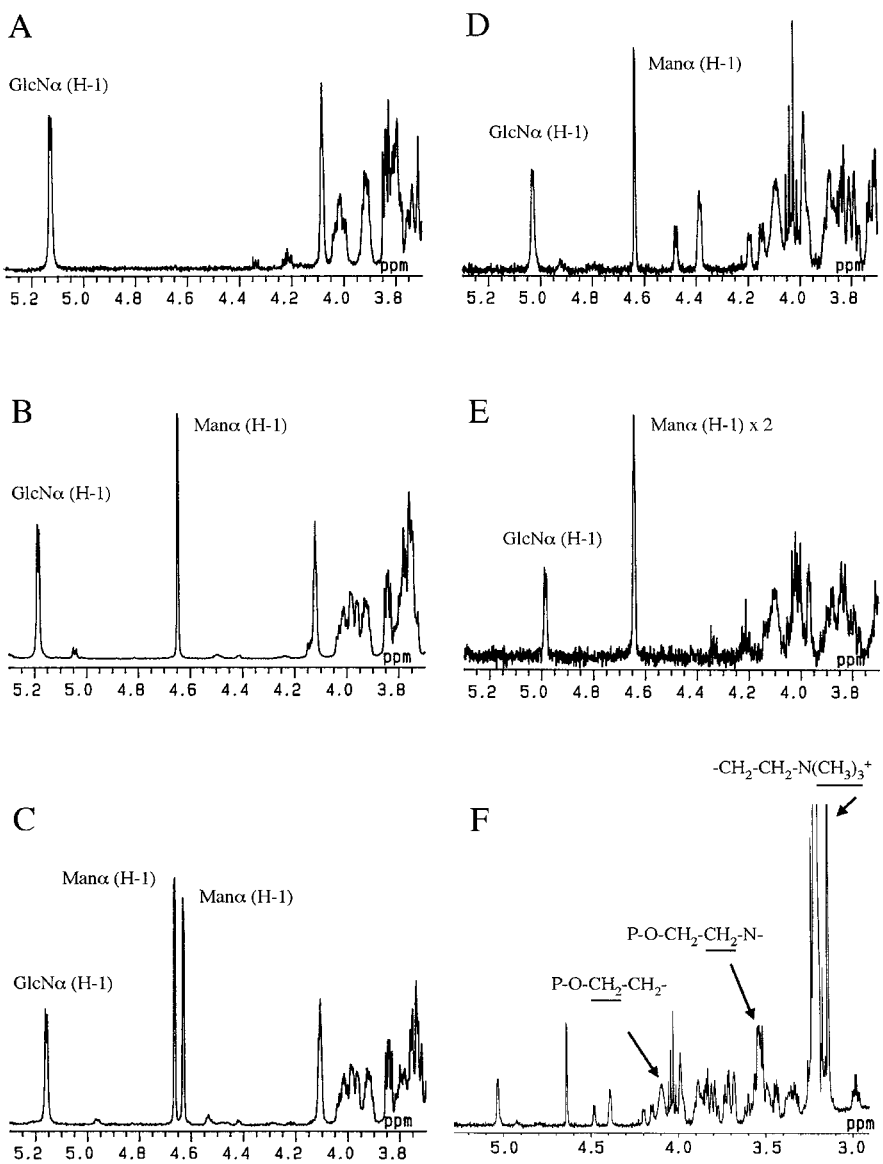


Figure 3 ^{1}H-NMR spectra of purified ZGLs

Anomeric proton regions of ZGLs: (**A**) ZGL1, (**B**) ZGL2, (**C**) ZGL3, (**D**) ZGL4, (**E**) ZGL5 and (**F**) choline phosphate region of ZGL4.

Table 4 Methylation analysis of purified ZGLs

Acremonium sp. ZGLs were N-acetylated and then subjected to methylation analysis. Treatment or not with HF is indicated by + HF and − HF respectively. The partially methylated alditol acetate derivatives of each glycolipid were analysed by GLC and GLC/MS, and the results are expressed relative to 1,6GlcN (=1.00). Appearance is indicated by +.

	Relative amount						
				ZGL4		ZGL5	
Linkage	ZGL1	ZGL2	ZGL3	− HF	+ HF	− HF	+ HF
1GlcNAc	+	−	−	−	−	−	−
1,6GlcNAc	−	1.00	1.00	+	1.00	1.00	1.00
1Man	−	0.86	1.03	−	−	−	−
1,6Man	−	−	1.21	−	1.30	0.98	2.14

4.66 p.p.m. (J 1.2 Hz), demonstrating 2 mol of α-mannose, and at 5.16 p.p.m. (J 3.7 Hz), demonstrating α-glucosamine, for ZGL3 (Figure 3C); at 4.64 p.p.m. (J 1.2 Hz), demonstrating α-mannose, and 5.03 p.p.m. (J 3.0 Hz), demonstrating α-glucosamine, for ZGL4 (Figure 3D); and at 4.64 p.p.m., demonstrating 2 mol of α-mannose, and 4.98 p.p.m. (J 3.7 Hz), demonstrating α-glucosamine, for ZGL5 (Figure 3E). In addition, in the ZGL4 and ZGL5 spectra three distinctive signals attributable to two methylene protons and one methyl proton of the choline phosphate were observed; these chemical shifts were 4.09 p.p.m. (2H, broad singlet), 3.51 p.p.m. (2H, broad singlet) and 3.14 p.p.m. (9H, singlet, 3 × Me) for ZGL4, and 4.10 p.p.m. (2H, broad singlet), 3.54 p.p.m. (2H, broad singlet) and 3.13 p.p.m. (9H, singlet, 3 × Me) for ZGL5 [36]. The characteristic choline phosphate regions of ZGL4 are shown in Figure 3(F). These observations also demonstrated the presence of a choline residue in ZGL4 and ZGL5. Enzymic hydrolysis of the ZGLs by α-mannosidase also showed the presence of an α-mannose residue at the terminal group of ZGL2 and ZGL3; however, PC-containing ZGLs such as ZGL4 and ZGL5 were not hydrolysed by α-mannosidase treatment (results not shown). These results suggest that PC is linked to the terminal mannose residue of both ZGL4 and ZGL5.

Sugar linkage and sequence analyses of ZGLs

Subsequently, in order to determine sugar linkages, the partially methylated alditol acetate derivatives of each NAc-ZGL were analysed by GLC and GLC/MS; the results are summarized in Table 4. Methylation analysis demonstrated the presence of 1-substituted *N*-acetylglucosamine (1,5-di-*O*-acetyl-3,4,6-tri-*O*-methyl-*N*-acetylglucosaminitol; 1GlcNAc) for NAc-ZGL1; 1-substituted mannose (1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol; 1Man) and 1,6-substituted *N*-acetylglucosamine (1,5,6-tri-*O*-acetyl-3,4-di-*O*-methyl-*N*-acetylglucosaminitol; 1,6 GlcNAc) for NAc-ZGL2; 1-substituted mannose, 1,6-substituted *N*-acetylglucosamine and 1,6-substituted mannose (1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylmannitol; 1,6Man) for NAc-ZGL3; 1,6-substituted *N*-acetylglucosamine for NAc-ZGL4; and 1,6-substituted *N*-acetylglucosamine and 1,6-substituted mannose for NAc-ZGL5. On methylation analysis of both NAc-ZGL4 and NAc-ZGL5, however, a terminal sugar residue was not detected. This suggested that PC is linked to the non-reducing end of the sugar chain.

In order to determine the linkage position of the terminal sugar residue in the sugar chain to PC, methylation analyses were performed after HF treatment. HF treatment was carried out under optimum conditions, whereby the phosphodiester linkage of PC, but not that of inositol 1-phosphate, could be cleaved. HF treatment after permethylation revealed the presence of a 1,6-substituted mannose residue in ZGL4 and ZGL5. These results suggested that PC is linked to the C-6 position of the terminal mannose reside in ZGL4 and ZGL5. In addition, to determine the substitution site of the inositol ring for glucosamine, NAc-ZGL1 was subjected to periodate oxidation. The possible glycosidic substitutions on inositol 1-phosphate with GlcNAc were at the C-2 to C-6 positions. The acetylated alcohol product obtained on periodate oxidation gave one peak on GLC, and its mass spectrum corresponded to that of erythritol [32,38]. This revealed that a glucosamine residue is linked to the C-2 position of the inositol ring (results not shown).

From these findings, the structures of the ZGLs were determined as follows: GlcN(α1-2)Ins1-*P*-1Cer for ZGL1, Man(α1-6)GlcN(α1-2)Ins1-*P*-1Cer for ZGL2, Man(α1-6)Man (α1-6)GlcN(α1-2)Ins1-*P*-1Cer for ZGL3, PC → 6Man(α1-6) GlcN(α1-2)Ins1-*P*-1Cer for ZGL4, and PC → 6Man(α1-6)Man (α1-6)GlcN(α1-2)Ins1-*P*-1Cer for ZGL5. These results confirmed that these fungal ZGLs are composed of novel constituents such as glucosamine and PC, and have an oligosaccharide structure similar to that of GPI-anchored protein.

MALDI-TOF/MS analysis

The putative structures of the purified AGL and ZGLs were confirmed by MALDI-TOF/MS analysis in the negative-ion mode, as shown in Figure 4. Their mass spectra had two different pseudomolecular ions because of the presence of two different fatty acid species, and both $[M-H]^-$ ions were in good agreement with the mass values calculated from the proposed structures: the $[M-H]^-$ ions of AGL at m/z 813.5 and 925.2 (Figure 4A) coincided with the mass values of compounds having 1 mol each of inositol, fatty acid (2-hydroxy-16:0 or -24:0), and sphingoid (t18:0) (see Table 2); those of ZGL1 at m/z 973.4 and 1087.5 (Figure 4B) coincided with the mass values of compounds having 1 mol each of glucosamine, inositol, fatty acid (2-hydroxy-16:0 or -24:0) and sphingoid (t18:0); those of ZGL2 at m/z 1136.5 and 1249.0 (Figure 4C) coincided with the mass values of compounds having 1 mol each of glucosamine, inositol, mannose, fatty acid (2-hydroxy-16:0 or -24:0) and sphingoid (t18:0); and those of ZGL3 at m/z 1299.1 and 1411.3 (Figure 4D) coincided with the mass values of compounds having 1 mol of glucosamine, 1 mol of inositol, 2 mol of mannose, and 1 mol each of fatty acid (2-hydroxy-16:0 or -24:0) and sphingoid (t18:0). The $[M-H]^-$ ion of ZGL4 at m/z 1414.7 (Figure 4E) was in agreement with the mass value of a compound having 1 mol each of inositol, mannose, PC and ceramide (2-hydroxy-24:0 as fatty acid and t18:0 as sphingoid), and that of ZGL5 at m/z 1577.2 (Figure 4F) coincided with the mass value of a compound having 1 mol each of inositol, PC and ceramide (2-hydroxy-24:0 as fatty acid and t18:0 as sphingoid), and 2 mol of mannose. However, the mass spectra of ZGL4 and ZGL5 exhibited unknown peaks which showed lower mass values (m/z 1355.5 in Figure 4E and m/z 1518.5 in Figure 4F), by 58 Da, than the calculated values. It is most likely that the loss of 58 mass units from the molecular ion in the laser desorption spectrum was caused by elimination of $CH_2=N(CH_3)_2$ from the PC group [39]. In fact, such an observation has been made in MALDI-TOF/MS analyses of phosphatidylcholine and PC of glycolipids. The putative structures of ZGLs were also identified by FAB/MS analysis in the negative-ion mode (results not shown).

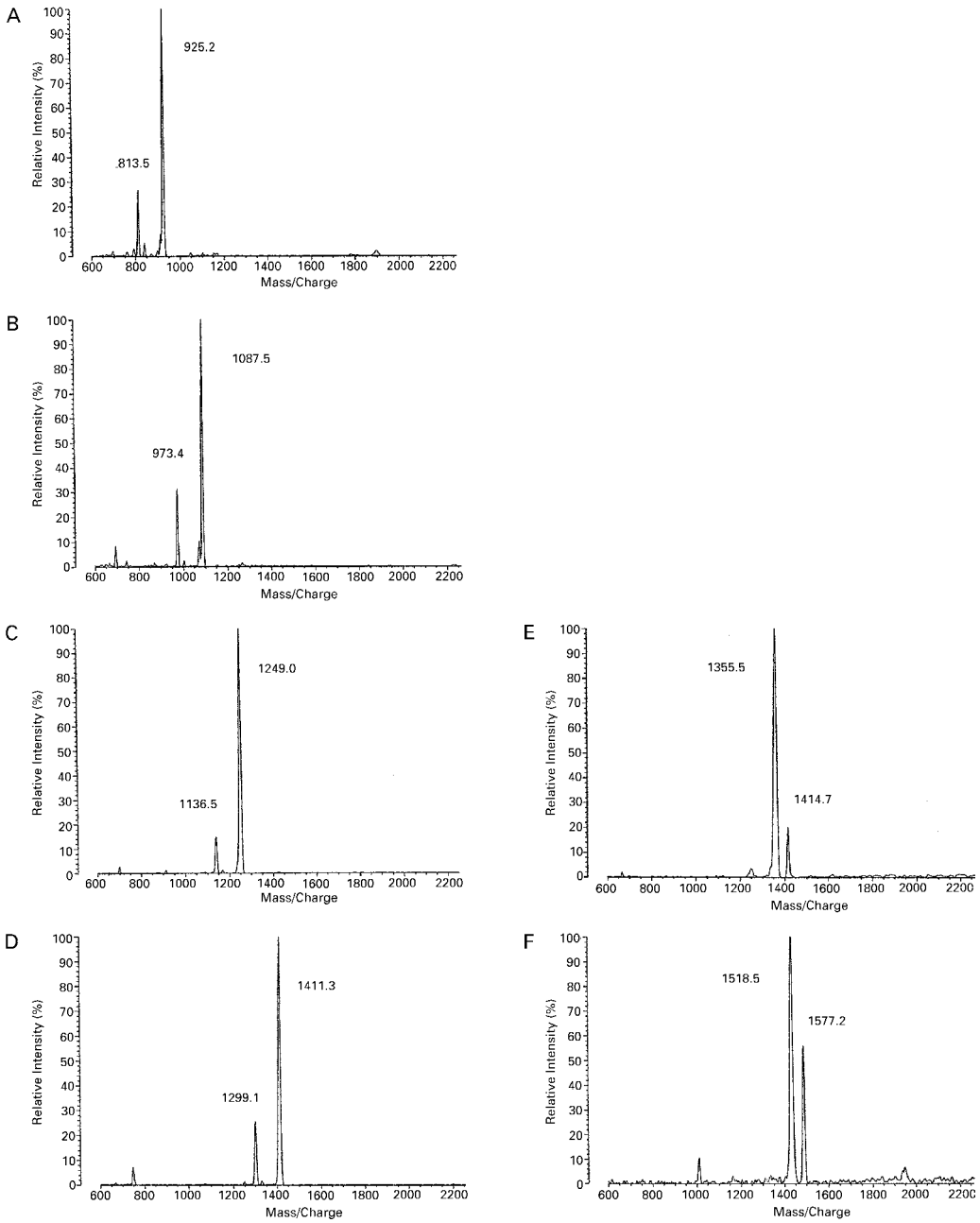


Figure 4 MALDI-TOF/MS spectra of purified GSLs

Analyses were performed in the negative-ion linear mode. Pseudomolecular ions are given in average masses. (**A**) AGL, (**B**) ZGL1, (**C**) ZGL2, (**D**) ZGL3, (**E**) ZGL4, (**F**) ZGL5. Major pseudomolecular ions of characteristic PC fragmentation were at *m/z* 1355.5 (**E**) and 1518.5 (**F**). Details are described in the text.

Cell death of cultured rice cells caused by fungal ZGLs

T. viride is known as a pathogen that infects rice plants. During the present investigation, we found that the ZGLs of *Acremonium* sp. seemed to be the same as the ZGLs from *T. viride* (IFO 30498), as judged by TLC (Figure 5). The NGL of *T. viride* was determined as glucosylceramide, which is common in fungal pathogens [14,15,35]. The ZGLs of *T. viride* seemed to be the same as those of *Acremonium* sp., although ZGL3 and ZGL5 were not found in the former (Figure 5). We analysed the ZGLs of *T. viride* by MALDI-TOF/MS and GLC/MS. The structures were confirmed to be the same as those of ZGL1, ZGL2 and ZGL4 of *Acremonium* sp. (results not shown). Therefore, in order to elucidate the function of these ZGLs present in plant pathogenic fungi, the defence responses of plants following inoculation of ZGLs into suspensions of cultured rice cells were investigated. At first, each suspension of cultured rice cells was inoculated with the fungal glucosylceramide that was reported to be a novel glycolipid elicitor for plants [14,15], human glucosylceramide from Gaucher's spleen cells that was reported not to induce cell death [14], MIPC prepared from *S. cerevisiae*, or a mixture of *Acremonium* sp. ZGLs. Assay of

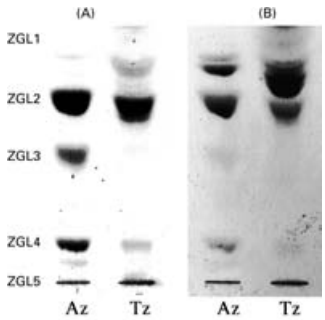


Figure 5 TLC of ZGLs from *T. viride*

The ZGL fraction from *T. viride* was analysed by TLC with propan-1-ol/water/15 M NH_4OH (75:25:5, by vol.; basic solvent), with visualization with orcinol/H_2SO_4 reagent (**A**) and Hanes–Isherwood reagent (**B**). Lane Az, ZGLs from *Acremonium* sp.; lane Tz, ZGLs from *T. viride*.

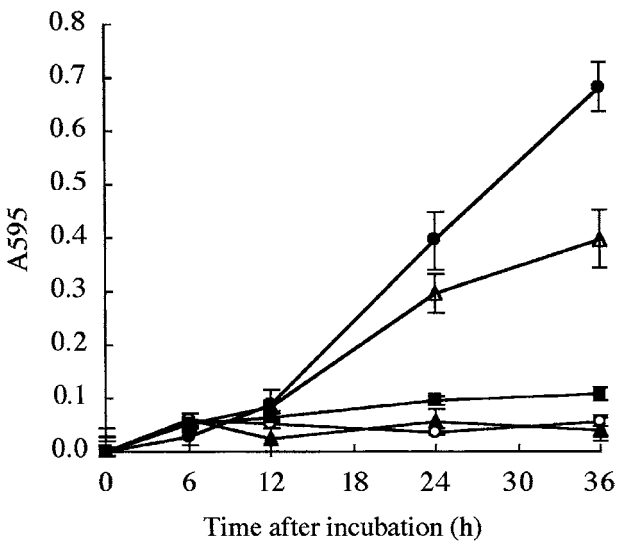


Figure 6 Cell death of cultured rice cells induced by treatment with *Acremonium* sp. ZGLs

Rice cells were treated with each GSL described below. Cell death was examined by determination of the absorbance at 595 nm of Evans Blue dye extracted from dead cells. ▲, DMSO (control); △, whole *Acremonium* sp. ZGLs (100 μg/ml); ●, glucosylceramide from fungi (50 μM); ○, glucosylceramide from Gausher's spleen (50 μM); ■, MIPC (50 μM). Each value is the average from three independent experiments; bars show S.D.

cell death was performed by determining the number of cells containing Evans Blue dye after incubation of the cells with the above GSL(s). As shown in Figure 6, cell death occurred on incubation with the fungal glucosylceramide for 24 h, with the amount of dead cells increasing gradually, whereas human glucosylceramide did not induce cell death. Interestingly, cell death was also induced by the addition of a mixture of ZGLs from *Acremonium* sp. after incubation for 24 h. However, MIPC did not cause cell death. The only significant difference between ZGLs and MIPC is the structure of the oligosaccharide: glucosamine binding to *myo*-inositol is present in ZGLs. To examine the relationship between structure and activity, the cell death of cultured rice cells was investigated further by using purified ZGL1, ZGL2 and ZGL4. As shown in Figure 7, ZGL2 induced

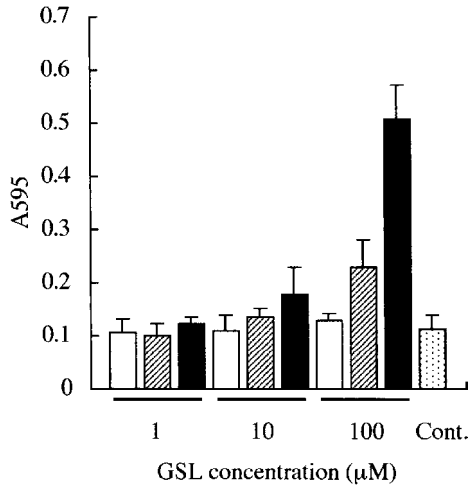


Figure 7 Effect of concentration of purified ZGLs on the cell death of cultured rice cells

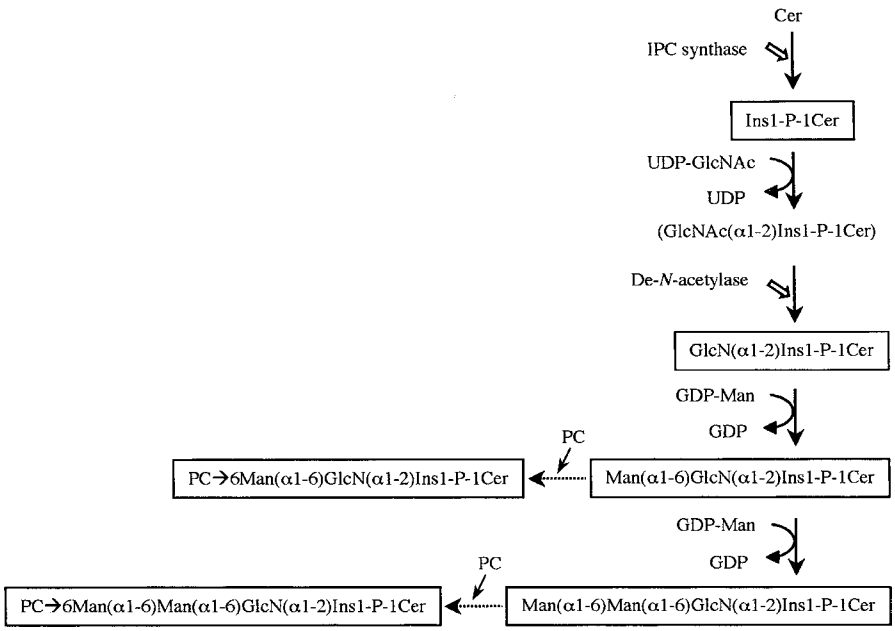
The responses (i.e. cell death) of cultured rice cells on the addition of purified ZGL1, ZGL2 or ZGL4 were determined after incubation with each ZGL (1, 10 and 100 μM) for 36 h. Open bars, ZGL1; hatched bars, ZGL2; closed bars, ZGL4; dotted bar, DMSO (control). Each value is the average for three independent experiments; bars show S.D.

the cell death of cultured rice cells; furthermore, the cell death-inducing activity of ZGL4, which contains PC, was approx. 2-fold higher than that of ZGL2. However, ZGL1 did not induce cell death, like MIPC. The results suggested that the Man-GlcN of the glycan in ZGLs is the key structure determining cell death, and that the presence of a PC residue promotes cell death-inducing activity.

DISCUSSION

Several studies have revealed that all fungi and plants have IPC as a common component of GSLs, with mannose added to IPC [5,19]. The biosynthetic pathway and the functional roles of fungal GSLs have been investigated in detail with *S. cerevisiae*, which synthesizes three types of GSLs: IPC, MIPC and $M(IP)_2C$. MIPC has also been found in various fungi and mushrooms [19,40–44]. The glycan motifs of these GIPCs have been identified as Manα1-2Ins [41,43] and Manα1-6Ins [42,44].

In the present work, analyses of GSLs in various filamentous fungi revealed the existence of a novel glycan structure. The novel glycan chains in ZGLs obtained from *Acremonium* sp. were GlcN(α1-2)Ins, Man(α1-6)GlcN(α1-2)Ins, Man(α1-6)Man(α1-6)GlcN(α1-2)Ins, PC $\rightarrow$ 6Man(α1-6)GlcN(α1-2)Ins and PC $\rightarrow$ 6Man(α1-6)Man(α1-6)GlcN(α1-2)Ins. To the best of our knowledge, such linkage structures of the glycan in GSLs have not been found previously in filamentous fungi, although Toledo et al. [45] demonstrated that the yeast form of the dimorphic mycopathogen *Sporothrix schenckii* had a similar glycan structure, Man(α1-3)Man(α1-6)GlcN(α1-2)Ins. Interestingly, glucosamine, not mannose, is linked to *myo*-inositol in GSLs of *Acremonium* sp., as in GSLs of *Sporothrix schenckii*. The core structure of their oligosaccharides, GlcN(α1-2)Ins, is the isomeric glycan of GlcN(α1-6)Ins, which is the common core structure of the GPI anchors that are widely distributed in eukaryotic cell membranes [25] and in the lipids of protozoans [46]. The biosynthetic pathways for mammalian and protozoan



Scheme 1 Putative biosynthetic pathway for GSLs in *Acremonium* sp.

GPIs are common: the core structure of GlcN-Ins is formed through the transfer of GlcNAc from UDP-GlcNAc to Ins of phosphatidylinositol, and subsequently de-N-acetylated. The biosynthetic pathway for GSLs from *Acremonium* sp. seems to be the same. The structural characterization of the *Acremonium* ZGLs leads us to propose a putative biosynthetic pathway, as shown in Scheme 1, involving the sequential addition of glucosamine, mannose and PC (or mannose phosphate and choline) to IPC.

There have been some reports that GSLs in invertebrate phyla such as the Annelida [36] and Nematoda [21,22] possess PC in the oligosaccharide moiety. Macromolecules containing PC have been detected in numerous parasitic nematode species. In particular, PC-containing GSLs, which are highly conserved among parasitic nematodes, were found to be biologically active in inducing the release of the pro-inflammatory monokine tumour necrosis factor α by human peripheral-blood mononuclear cells, and their activities were found to be dependent on the presence of the PC substituent [21]. Furthermore, PC was found in lipoteichoic acid [47] and the cell wall associated with teichoic acid of *Streptococcus pneumoniae* [48], in the lipopolysaccharide of *Haemophilus influenzae* [49], and in the glycoglycerolipid of *Mycoplasma fermentans* [20]. PC-containing glycoglycerolipids of *Mycoplasma fermentans* were reported to be one important factor associated with rheumatoid arthritis [50]. A striking finding in the present work is the presence of PC-containing GSLs in fungi. There have been no previous reports of the existence of glycolipid-conjugated PC in fungi, and this is the first study revealing the occurrence of GSLs that include both PC and inositol phosphate in organisms. PC-containing GSLs may also have important roles in the fungal infection of mammals, since PC-containing molecules have been found in many pathogens [20,47–50] and parasitic nematodes [21–23], and have been shown to modulate host–pathogen interactions [24].

The molecular roles of fungal GSLs are still little known. However, it has been reported that a fungal cerebroside from *Magnaporthe grisea* exhibited elicitor activity and induced defence responses in plants, whereas mammalian cerebroside did not [14]. This observation indicates that plants can specifically recognize the structure of fungal cerebrosides in order to protect themselves. The structural features of fungal cerebroside required for elicitor activity were reported to be the 4*E*- and 8*E*-double bonds, and the methyl group at the C-9 position in the sphingoid base moiety [14]. In particular, the characteristic methyl group at the C-9 position in the sphingoid base, which is present in fungi but absent in mammals, is important for the elicitor activities [14,15]. Interestingly, the common ZGLs present in *Acremonium* sp. and *T. viride* stimulated the cell death of cultured rice cells, although their sphingoid bases did not have the above structural features. As fungal MIPC and GlcN-IPC (ZGL1) did not induce cell death, we conclude that Man-GlcN in the glycans of ZGLs is the key structure for the cell death-inducing activity. Moreover, as PC-containing ZGLs were more active in the stimulation of cell death than non-PC-containing ZGLs, it seems that PC has a significant effect in causing cell death. The fact that ZGLs are characteristically distributed in plant pathogenic fungi such as *T. viride* suggests that they play important roles in the infection of plants.

Nuclear DNA cleavage appears to be a feature of programmed cell death in animals. It has been reported that plant DNA cleavage was also observed during hypersensitive cell death, triggered by infection with virus, bacteria or fungi [51], and in death caused by pathogen-related components such as flagelline, glucan and chitin [14,15,34,51]. Several lines of evidence suggest that the cell death of plant cells occurs via the activation of a programmed cell death pathway as the hypersensitive response. However, DNA cleavage was not detected after treatment with ZGLs and, interestingly, an oxidative burst did not occur following inoculation of ZGLs (results not shown). It is important to investigate whether these ZGLs cause other defence responses in plant cells, such as the induction of phytoalexins, pathogenesis-related protein, etc. [14,15,34,51]. Further studies on hypersensitive responses caused by fungal ZGLs are now in progress.

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